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Effect of Water Clover (Marsilea crenata) Leaf Extract on Estrogen Receptors-β in Skin Aging

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A B S T R A C T

Extrinsic and intrinsic factors influence skin aging. Hormonal changes, especially estrogen, significantly affect intrinsic skin aging. Decreased circulating estrogen levels reduce skin collagen content and skin elasticity. Isoflavones in *Marsilea crenata* (MC) leaf are active substances containing compounds that mimic estrogen. This study aims to analyze MC leaf extract against the estrogen receptor (ER)-β. The sample for this research was female Wistar Rats (*Rattus norwegicus*). All of them were 12 months old, with their weight was between 350 to 550 grams divided into five groups. P1 P2 and P3 were grouping with MC leaf extract administration in sequential doses 20 mg/kg BW, 30 mg/kg BW, and 40 mg/kg BW. At the same time, P4 was a positive control group, and P5 was a negative control group. The independent variable was *M.crenata* leaf extract. The dependent variables were (ER)-β expression and dermal thickness. The data analysis utilized the one-way analysis of variance (ANOVA) showed significant differences ($p < 0.05$). Each group showed significant results, and group P2 showed the highest expression of (ER)-β and dermal thickness. The result showed that there were significant correlations between both variables ($P<0.05$). This research has proved that water clover extract could become an alternative treatment in the future for skin aging. However, further research should find a proper dose for human consumption.

INTRODUCTION

Skin aging is a degenerative process influenced by intrinsic factors (genetic and hormonal) which occur together with extrinsic factors (sun, heat, pollution, smoking) (Chung, Cho, and Kang, 2004; Assaf, Adly, and Hussein, 2010; Garg, Khurana and Garg, 2017). Hormones such as estrogen, testosterone, dehydroepiandrosterone (DHEA), melatonin, cortisol, growth hormone (GH) decrease with age (Yaar, 2006; Zouboulis and Makrantonaki, 2006). Decreased circulating estrogen levels may lead to skin aging (Laure *et al.*, 2008; Thompson and Maiibach, 2010; Kohl *et al.*, 2011; Yaar and Gilchrest, 2012; Tobin, 2017).

Estrogen is useful in treating aging skin after six months of treatment in premenopausal women with skin aging symptoms. It improves skin elasticity and wrinkle depth is (Liu et al., 2019). Phytoestrogens bind to REα and REβ – more bound to REβ. After binding with ligands, these receptors can move from the cytoplasm to the nucleus, bind and influence the transcription control regions of small DNA or RNA, and express specific genes. Furthermore, steroids can bind to cell surface receptors, promote the formation of cytoplasmic cyclic nucleotides and related protein kinases, which in turn, through transcription factors control the expression of target genes. Therefore, phytoestrogens can influence estrogen-regulated

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processes. It affects the induction of sex hormone binding globulin (SHBG) and aromatase inhibition. (Sirotkin and Harrath, 2014).

Marsilea crenata (MC) or water clover is a group of *salviniales* living wild in aquatic environments such as ponds, rice fields, lakes, and swamps. There are isoflavones, a part of flavonoids, in MC leaves. Isoflavones are active substances that contain compounds that mimic estrogen. It can activate ER in mammals, so they are often called phytoestrogen isoflavones (Titisari *et al.*, 2016). Phytoestrogens are non-steroidal organic phytochemicals. There are several types in the phytoestrogens class: lignans, stilbene, coumestans, coumarin, dihydrochalcone, triterpenoids, and flavones. In humans, phytoestrogen activity is similar to estrogen. Phytoestrogen potential is estimated to be lower than 17-β-estradiol. The action mechanism of phytoestrogens is similar to structural 17-β-estradiol. This component binds to both ER-α and ER-β (Kapuscinska, Nowak, and Mickiewicz, 2015). The background of this research because there has not been a similar study regarding MC leaf extract's effect on ER- β on skin aging.

Table 1. Predicted compounds of 96% ethanol extract of *M.crenata* from Benowo District, Surabaya inmethanol solvent (Ma'arif, Agil and Widyowati, 2019).

No	Rt (min)	% Area	Measured m/z	Molecular Formula	Proposed Metabolite	Activity
1	0.201	0.0228	124.9790	CH3NO6	Unknown	-
2	1.535	2.4313	235.1423	C10H21NO5	4-(3-Hydroxypropyl)-4-nitro-1,7-heptanediol	-
3	2.232	0.1510	179.1315	C11H21NO7	2[(tertButoxycarbonyl)amino-2-deoxy-D glucopyranose	-
4	2.518	1.5144	293.1479	C12H23NO7	Methyl 6-deoxy-6-({[(2-methyl-2-propanyl)oxy]carbonyl}amino)-β-D-glucopyranoside	-
5	3.799	1.4856	327.1314	C15H21NO7	Methyl (3,4,5-triethoxy-2-nitrophenyl)acetate	-
6	4.427	1.4055	187.0642	C5H15N3Cl2	4-Hydrazinopiperidine dihydrochloride	-
7	4.610	0.3629	162.0321	C9H6O3	3-Hydroxy-2H-chromen-2-one (3 hydroxycoumarin)	Competitive inhibition of human recombinant DAAO [23].
8	4.896	0.1836	373.1375	C20H24N3SCl	Prochlorperazine	Analgesics [24], antiemetics [25]
9	5.228	0.9215	359.0997	C13H18N5O5Cl	Ethyl 4-[3-(4-chloro-3-nitro-1H-pyrazol-1-yl)propanoyl]-1-piperazinecarboxylate	-
10	5.445	0.0257	475.2990	C33H37N3	4-{Bis[4-(1-pyrrolidinyl)phenyl]methyl}-N,N-dimethyl-1-naphthalenamine	-
11	5.628	0.9906	343.1051	C10H21N3O8S	1-Azido-1-deoxy-2,3-bis-O-methoxymethyl)-5-O-(methylsulfonyl)-D-ribitol	-
12	5.845	0.6908	550.0951	C24H22O15	Quercetin-3-(6"-malonyl)-Glucoside	Antioxidant and antiatherogenic protective [26]
13	6.177	1.0895	498.1166	C25H22O11	4-(1,3-Benzodioxol-5-yl)-6-hydroxy-1-oxo 1,3-dihydronaphtho [2,3-c]furan-5-yl hexopyranoside	-
14	6.577	0.3205	534.1013	C24H22O14	2(3,4Dihydroxyphenyl)-5-hydroxy-4-oxo-4H chromen-7-yl 6-O (carboxyacetyl)-β-Dglucopyranoside "luteolin 7-O-(6-O-malonyl-β-D-glucoside"	-
15	6.908	0.2713	219.1625	C14H21NO	1-[1-(4-Methoxyphenyl)cyclohexyl]methanamine	-
16	7.206	2.0878	196.1105	C11H16O3	1-carboxy-3-hydroxyadamantane	-

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This study aims to determine MC leaf extract's effects for ER-beta on skin aging so that the research results will contribute positively to anti-aging dermatology.

METHOD

Section 1. Materials& Instruments Research

Material Research

- The experimental animal, female Wistar (*Rattus norwegicus*) rats, 12 months of age, and body weight between 350-550 grams were obtained through the Laboratory of the Institute of Tropical Disease, Airlangga University, Surabaya, and carried out for one week of acclimation before the intervention.
- Standard forage
- Clover leaf extract (*M.crenata*) is obtained from clover leaves extracted from 96% ethanol, which is made in the Pharmacy Laboratory of Airlangga University
- Estradiol Tablets
- Ketamine 50 mg / kg BW
- Materials for immunohistochemical examination
- Material for HE examination.

The instruments were:

- individual mouse cages
- rat fixator, razors
- 0.5 cm biopsy punch
- Tanita brand scale
- ruler, books and stationery
- some tools for preparation
- microscope**.**

Section 2. The Research Design

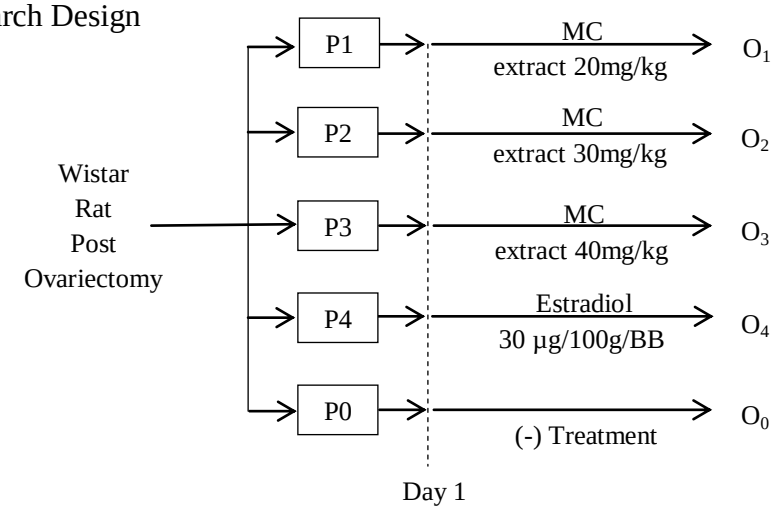

Figure 1. Research Plan

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The sample in this study was female Wistar rats (*Rattus norwegicus*) aged 12 months with weight between 350-550 grams which were ovariectomized by treatment: (1) was given 20 mg/kg BW MC extract (P1); (2) given 30 mg/kg BW MC extract (P2); (3) given 40 mg/kg BW MC extract (P3); (4) were given 30 µg / 100g BW estradiol tablet (P4); (5) dan no treatment (P0). This study's design used four rats in each group plus two rats in anticipation of rats getting sick or dying during treatment. The number of rats in each group was six rats, and the total number of rats for the five treatment groups needed is 30 rats. Samples taken were randomized into groups P1, P2, P3, P4, and P0. This study used a sample of Wistar rats (*Rattus norwegicus*) that met the inclusion and exclusion criteria of the study: (1) Inclusion criteria: All healthy and female Wistar rats (*Rattus norwegicus*), 12 months of age, 350-550 grams of weight; (2) Exclusion criteria: Rats had skin disorders, were sick and died.

Research using female Wistar rats was conducted at the Animal Stem Cell Laboratory of the Institute of Tropical Disease, Airlangga University, Surabaya. The process of extracting MC leaves was made at the Pharmacy Laboratory of HangTuah University, Surabaya. Immunohistochemical examinations were carried out at the Anatomical Pathology Laboratory, Faculty of Medicine, Brawijaya University. The research took place from January-September 2020. The research procedure:

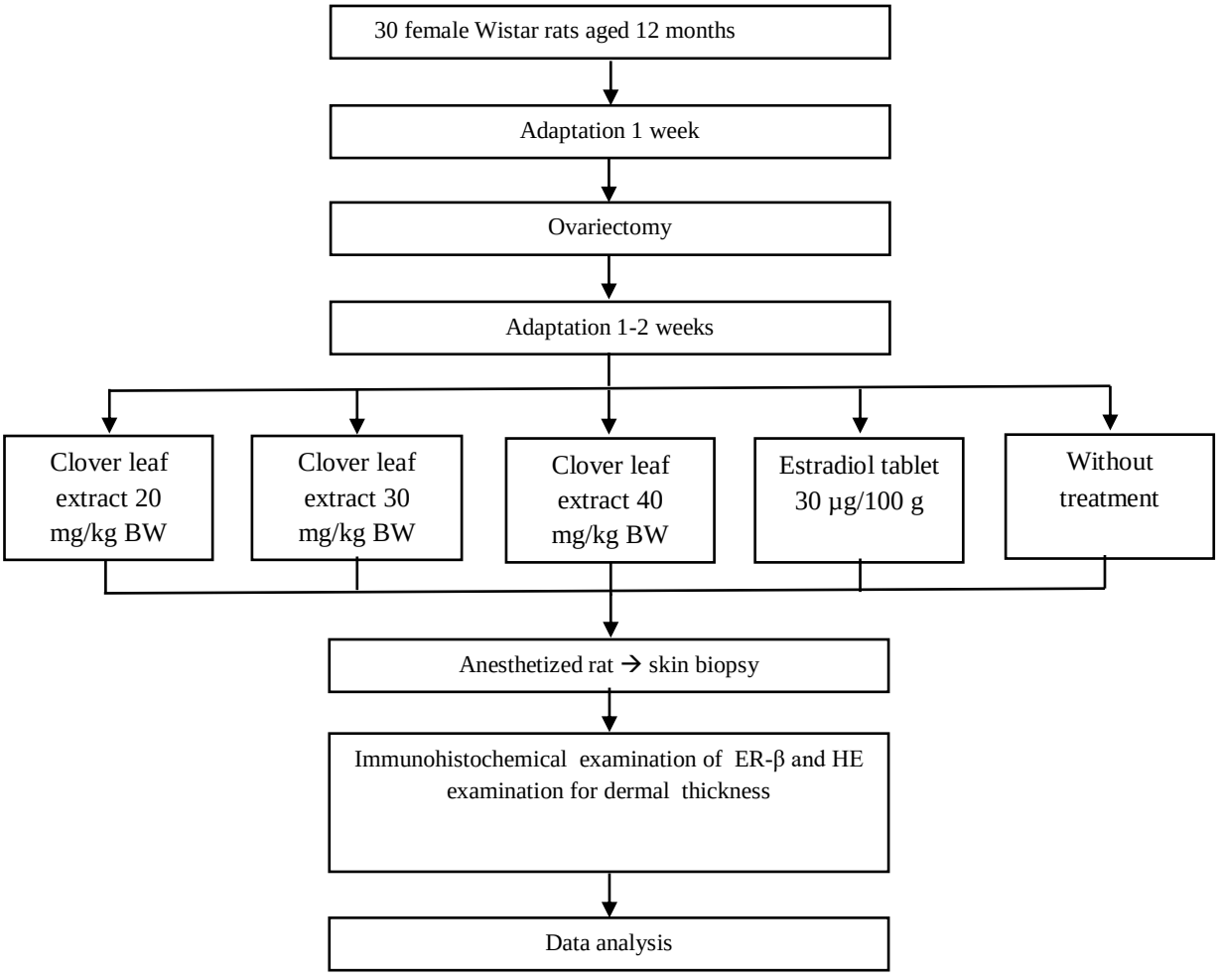

Figure 2. Research Procedure

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Data were collected from the research status, then performed cleaning, editing, and coding. Then data analysis utilized Statistical Package for the Social Sciences (SPSS) data format version 20.0 (SPSS, Inc., Chicago, Illinois). The statistical test used was the normality test with the Shapiro-Wilk test because the sample number was smaller than 30 per group, and the data were normally distributed ($p > 0.05$). Parametric statistical tests with the Pearson Correlation test analyzed homogeneous data for correlation analysis.

RESULTS

In this study, we treated post ovariectomy female Wistar rats with MC leaf extract with three different doses, group P1 at a dose of 20 mg/kg BW, and P2 at a 30 mg/kg BW dose, and P3 at a dose of 40 mg/kg BW. Another group, P4, was given estradiol tablets 30 µg / 100 g BW as a positive control, while the last group, P0, was not given any treatment. Several rats died during the treatment in the treatment group with MC leaf extract (1 group P1, three groups P3), one rat in the treatment group with estradiol tablets, and three rats in the control group

Table 1. Research Results

Group	Mean Expression ER-β±SD	Mean of Dermal Thickness±SD
P1 MC 20 mg/kgBW	7 ± 1,414	2,8575 ± 0,336
P2 MC 30 mg/kgBW	11,25 ± 1,258	3,445 ± 0,316
P3 MC 40 mg/kgBW	7,5 ± 1,291	2,1425 ± 0,121
P4 Estradiol 30 µg/100 g BW	9,25 ± 1,258	2,9225 ± 0,244
P0 Without Treatment	3 ± 1,826	1,9625 ± 0,115

Note: P1 : administration of MC leaf extract 20 mg/kgBW
P2 : administration of MC leaf extract 30 mg/kgBW
P3 : administration of MC leaf extract 40 mg/kgBW
P4 : administration of Estradiol 30 µg/100 g BW
P0 : group 0 Without treatment

P2 showed the highest ER-β expression and dermal thickness in the three treatment groups with MC leaf extract. ER-β expression in the P3 group was not as high as the P2 group but was still higher than the P1 group. In contrast, the dermal thickness in the P1 group was not as high as the P2 but was still higher than the P3 group. ER-β expression and dermal thickness in the P4 group were higher than the P1 and P3 groups but lower than the P2 group. The P0 group – without any treatment – showed the lowest ER-β expression and dermal thickness than other treatment groups (P1, P2, P3, and P4). The results showed that MC leaf extract affected ER-β and dermal thickness at the end of the study.

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There were significant differences in the ER-β expression and dermal thickness between groups with analysis using One-way ANOVA. The difference in mean ER-β expression and dermal thickness was significant when the p-value < 0,05. From the test results, the p-value was 0.000, so there was a significant difference in ER-β expression and dermal thickness between groups. Pearson correlation test analyzed a correlation between ER-β expression and dermal thickness of Wistar rats after ovariectomy. The results of this test showed that Sig. (2-tailed) between dermal thickness and ER-β was 0.000 < 0,05, which means there was a significant correlation between both variables.

DISCUSSION

This study aims to prove the effect of MC extract on increasing ER-β expression on skin aging. In this study, the average ER-β expression and dermal thickness increased after being treated with MC leaf extract and estradiol tablets. Studies on MC leaf extract in skin aging are still rare; however, there were studies on phytoestrogens from other sources. This study's result is in line with research conducted by Laswati et al. (2016), which uses phytoestrogens from tomatoes. The ER-β of post ovariectomy rats without phytoestrogens was lower than with phytoestrogens (Laswati *et al.*, 2016). One of the elements in *M.crenata* is an isoflavone. Isoflavone has a chemical structure similar to 17 –β estradiol – isoflavone can bind to estrogen receptors. Isoflavones act as agonists of estrogen receptors, but isoflavones have less activity than 17 –β estradiol. However, the endogenous estrogen levels in the body influence isoflavone's effect (Pilsakova et al., 2010).

A study done by Pilkasova et al. (2010) reported that isoflavones could inhibit the activity of 5α-reductase. 5α-reductase catalyzed the conversion of testosterone to 5α-dihydrotestosterone and aromatase P450. Isoflavones mediated the conversion from testosterone to estradiol. A low concentration of isoflavones inhibits aromatase activity. High isoflavone levels increase this enzyme activity (Almstrup et al., 2002). Isoflavones bind to the sex hormone binding globulin (SHBG) and stimulate its synthesis (Berrino et al. 2001). Alterations in SHBG concentration may yield changes in circulating steroid hormones (Pilsakova et al., 2010).

In another study by Mahmoud et al. (2015) reported that genistein, a component of phytoestrogens, can increase ER-β expression as the dose of genistein increases through the mechanism of phosphorylation, nuclear translocation, and ER-β transcription (Mahmoud et al., 2015). In this study, the P3 group showed ER-β expression not higher than the P2 group but higher than the P1 group. The ER-β expression in the P4 group (estradiol tablets) was higher than the P1 and P3 groups. These results align with the theory that states that phytoestrogen activity is lower than 17-β-estradiol (Pilsakova, Riecansky, and Jagla, 2010).

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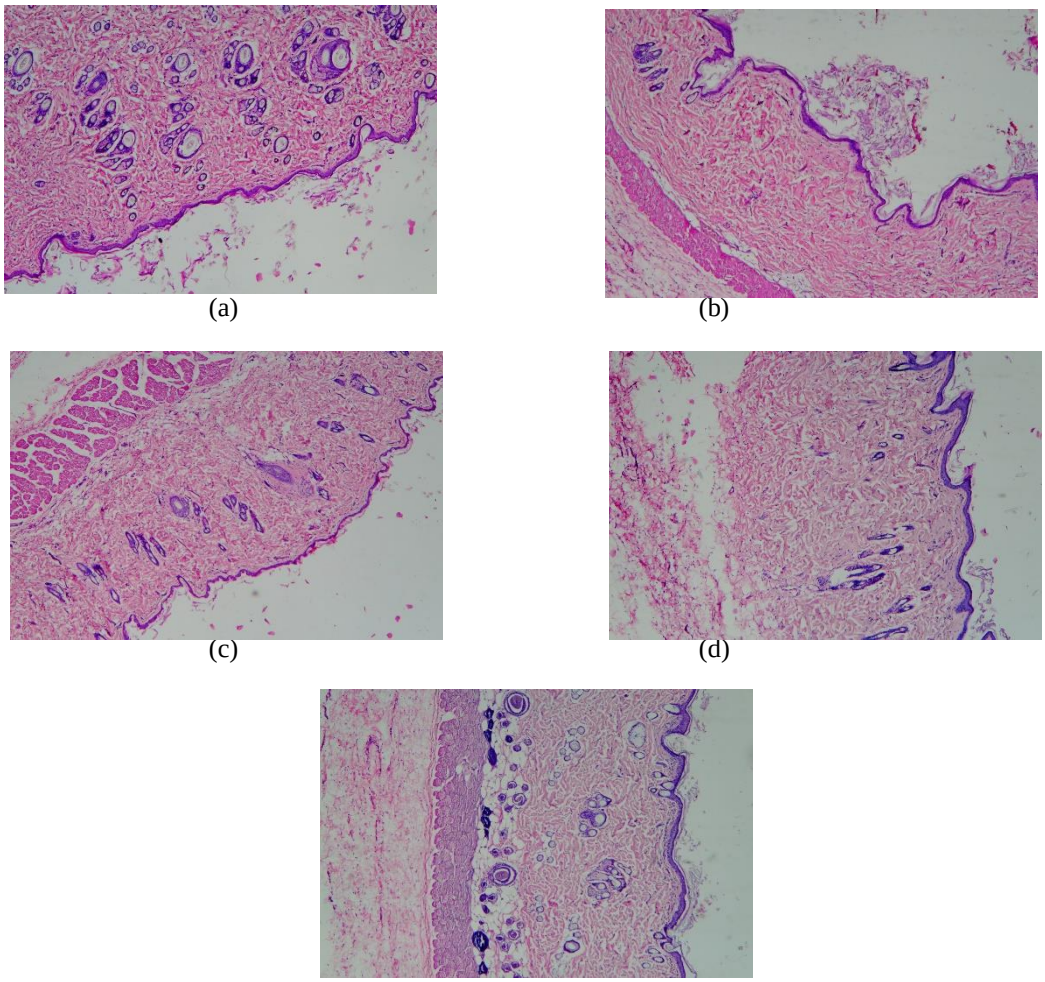

Figure 3. Skin cross-section (a) group P1, (b) group P2, (c) group P3, (d) group P4, (e) group P0

Apart from ER-β expression, this study also examined dermal thickness. The skin is a target organ for estrogen receptor beta hormone so that when there is a decrease in estrogen with aging, it will affect the skin. Manifestations of decreased estrogen in the skin include thinning of the skin, reduced collagen, dry and thin skin, and decreased skin vascularity (Liu *et al.*, 2019; Carneiro *et al.*, 2020). The skin aging mechanism occurs due to an increase in free radicals / ROS, which causes activation of cytokine and growth factor receptors on the surface of keratinocytes and dermal cells and initiates downstream signal transduction pathways (Chung, Cho, and Kang, 2004; Alam and Havey, 2010). Several signaling pathways can further increase inflammatory cytokines (IL-1, IL-6, TNF-α) and collagen-degrading enzymes (MMP) (Chung, Cho, and Kang, 2004; Baumann, Saghari, and Weisberg, 2009). An increase in MMP causes a decrease in the dermis quality, including a decrease in dermal thickness due to a decrease in the amount of collagen (Kavitha and Thampan, 2008; Baumann, Saghari, and Weisberg, 2009; Mizukoshi *et al.*, 2015). In this study, there was an increase in dermal thickness in the treatment group than in the control group that was not treated either with MC leaf extract or estradiol tablets. The optimal

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dose of MC leaf extract is 30 mg/kg BW/day. In this study, the P3 group with a 40 mg/kg BW dose found that the dermal thickness was lower than P1 20 mg/kg BW and P2 30 mg/kg BW and estradiol tablets. This result is similar to studies by Akyun, Fajariyah, and Mahriani (2019), which uses phytoestrogens in the form of black soybean ethanol extract. A study conducted by Akyun, Fajariyah, and Mahriani (2019) found that this plant extract can increase dermal thickness at a dose of 0.31 g / ml/day rather than the higher dose of 0.63 g /ml/day. The conclusion was 0.31 g / ml/day as an optimal dose (Akyun, Fajariyah, and Mahriani, 2019). Isoflavones can reduce wrinkling and skin thinning through collagen synthesis and decrease collagen degradation (Liu *et al.*, 2019). Another study by Moraes et al. (2009) showed an increase in dermal thickness with isoflavones still lower than the topical estrogen observed for six months.

CONCLUSIONS

Administration of water clover (M.crenata) leaf extract for four weeks increases the expression of β estrogen receptors and the dermal thickness in the Wistar post ovariectomy. MC leaf extract administration at a 30 mg/kg BW dose is optimal for improving skin aging more considerably than the 40 mg/kg BW/day dose. Finally, we can conclude that phytoestrogens in water clover have positive effects as estrogen replacement therapy.

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